IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: **NI et al.**

Application Serial No.: 09/042,583 Art Unit: 1646

Filed: March 17, 1998 Examiner: Kaufman, C.

For: Death Domain Containing Receptor 5 Atty Docket No: **1488.1310002/EKS/EJH**

DECLARATION OF JIAN NI, REINER L. GENTZ, GUO-LIANG YU AND CRAIG A. ROSEN UNDER 37 C.F.R. § 1.131

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

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1. We are named inventors of the captioned application, which is assigned to Human Genome Sciences, Inc. (HGS), a Delaware Corporation that has a principle place of business at 9410 Key West Avenue, Rockville, Maryland, USA. We are present or former employees of HGS. This declaration is made to establish possession and appreciation of the sequences described in the above-captioned application, in the United States, prior to March 12, 1997.

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8/2/03
Date

Jian Ni

Date

Reiner L. Gentz

Date

Guo-Liang Yu

Date

Craig A. Rosen

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Date	Jian Ni
7/30/2003 Date	Reiner L. Gentz
Date	Guo-Liang Yu
Date	Craig A. Rosen

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8/4/03 Date	Guo-Liang Yu
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Date	Jian Ni
Date	Reiner L. Gentz
Date	Guo-Liang Yu
Date 8/5/03	Craig A. Rosen

HLYBX88

Date Sequenced:

CTGAG ACTCTGAGAC AGTGCTTCGA TGACTTTGCA

HLYBX88

GACTTGGTGC CCTTTGACTC CTGGGAGCCG CTCATGAGGA AGTTGGGCCT CATGGACAAT
GAGATAAAGG TGGCTAAAGC TGAGGCAGCG GGCCACAGGG ACACCTTGTA CACGATGCTG
AT

Author: Jian Ni
Date:
Normal
TO:
TO:
TO:
TO:
Subject: TR7 patent
---------- Message Contents ----------
Attached please find informations for TR7 (HLYBX88) patent.

databank% type HLYBX88XX.publish

Signal peptide 1-51, transmembrane domain: 185-208. 52-184 are extrcellular domain. 209 to 411 is intracelluar domain, which contain death domain (Italic, 324-391).

```
        10                  30                  50
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        70                  90                  110
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       130                 150                 170
CCTACCGCCATGGAACAACGGGGACAGAACGCCCCGGCCGCTTCGGGGGCCCGGAAAAGG
          M  E  Q  R  G  Q  N  A  P  A  A  S  G  A  R  K  R
       190                 210                 230
CACGGCCCAGGACCCAGGGAGGCGCGGGGAGCCAGGCCTGGGCCCCGGGTCCCCAAGACC
H  G  P  G  P  R  E  A  R  G  A  R  P  G  P  R  V  P  K  T
       250                 270                 290
CTTGTGCTCGTTGTCGCCGCGGTCCTGCTGTTGGTCTCAGCTGAGTCTGCTCTGATCACC
L  V  L  V  V  A  A  V  L  L  L  V  S  A  E  S  A  L  I  T
       310                 330                 350
CAACAAGACCTAGCTCCCCAGCAGAGAGCGGCCCCACAACAAAAGAGGTCCAGCCCCTCA
Q  Q  D  L  A  P  Q  Q  R  A  A  P  Q  Q  K  R  S  S  P  S
       370                 390                 410
GAGGGATTGTGTCCACCTGGACACCATATCTCAGAAGACGGTAGAGATTGCATCTCCTGC
E  G  L  C  P  P  G  H  H  I  S  E  D  G  R  D  C  I  S  C
       430                 450                 470
AAATATGGACAGGACTATAGCACTCACTGGAATGACCTCCTTTTCTGCTTGCGCTGCACC
K  Y  G  Q  D  Y  S  T  H  W  N  D  L  L  F  C  L  R  C  T
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Q  C  E  E  G  T  F  R  E  E  D  S  P  E  M  C  R  K  C  R
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C  V  H  K  E  S  G  I  I  I  G  V  T  V  A  A  V  V  L  I
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V  A  V  F  V  C  K  S  L  L  W  K  K  V  L  P  Y  L  K  G
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A  E  D  N  V  L  N  E  I  V  S  I  L  Q  P  T  Q  V  P  E
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Q  E  M  E  V  Q  E  P  A  E  P  T  G  V  N  M  L  S  P  G
       970                 990                1010
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V  P  A  N  E  G  D  P  T  E  T  L  R  Q  C  F  D  D  F  A
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D  L  V  P  F  D  S  W  E  P  L  M  R  K  L  G  L  M  D  N
      1150                1170                1190
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E  I  K  V  A  K  A  E  A  A  G  H  R  D  T  L  Y  T  M  L
      1210                1230                1250
ATAAAGTGGGTCAACAAAACCGGGCGAGATGCCTCTGTCCACACCCTGCTGGATGCCTTG
I  K  W  V  N  K  T  G  R  D  A  S  V  H  T  L  L  D  A  L
      1270                1290                1310
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E  T  L  G  E  R  L  A  K  Q  K  I  E  D  H  L  L  S  S  G
      1330                1350                1370
AAGTTCATGTATCTAGAAGGTAATGCAGACTCTGCCATGTCCTAAGTGTGATTCTCTTCA
K  F  M  Y  L  E  G  N  A  D  S  A  M  S  *
      1390                1410                1430
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      1450                1470                1490
AGTAGGAAAGTGCCACAATTGTCACATGACCGGTACTGGAAGAAACTCTCCCATCCAACA
      1510                1530                1550
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TGAATGTGATAATAAGGACACTATGGAAAAAAAAAAAAA
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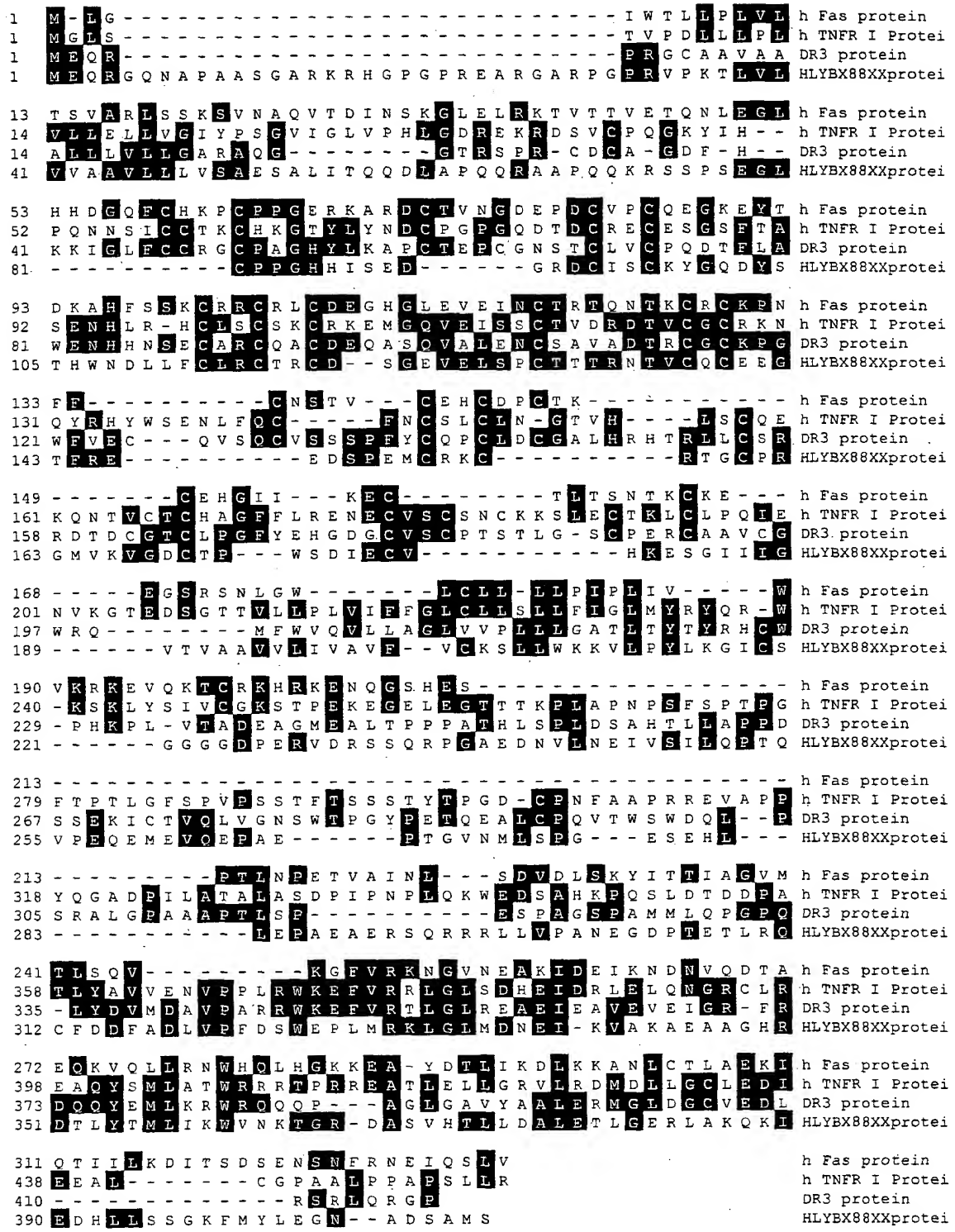

Decoration 'Decoration #1': Shade (with solid black) residues that match the Consensus exactly

Query Information

Cell, Vol. 76, 959–962, March 25, 1994, Copyright © 1994 by Cell Press

The TNF Receptor Superfamily of Cellular and Viral Proteins: Activation, Costimulation, and Death

Minireview

Craig A. Smith, Terry Farrah, and Raymond G. Goodwin
Departments of Biochemistry and Molecular Biology
Immunex Corporation
Seattle, Washington 98101

Tumor necrosis factor (TNF) seems always to have enjoyed a rather conspicuous visibility in biomedical research. With historical roots in the century-old phenomenon of bacterial-induced hemorrhagic necrosis of tumors, TNF—or, rather, its two homologous forms, TNFα and LTα (lymphotoxin, TNFβ)—were finally molecularly cloned in 1984, among the very first cytokines to be so unambiguously defined. Although TNFα and LTα, classically the respective products of activated macrophages and T cells, can indeed kill many transformed lines, these functionally similar and extraordinarily pleiotropic cytokines are today viewed as primary mediators of immune regulation and the inflammatory response, closely linked to the development of disease. The crucial involvment of TNF, for example, in septic shock, some autoimmune disorders, and graft-host disease is well established (see Beutler, 1992).

Since the cloning of two distinct but structurally homologous receptors for TNF, p75 and p55 (each of which binds both ligands), the past 3 years have witnessed the rapid emergence of two superfamilies, of which the TNFs and their receptors are only representatives (Farrah and Smith, 1992; Suda et al., 1993; Smith et al., 1993). To date, 12 receptors have been identified (Figure 1) with which we can associate some eight TNF-related cytokines (Figure 2). The distinctive but overlapping cellular responses their interactions produce clearly define developmental and regulatory networks involving cells of the lymphoid, hematopoetic, and other lineages. In this minireview we make no attempt to discuss individual members comprehensively and instead highlight emerging global characteristics that distinguish them from other cytokine families: structure, biological networks, and the intriguing ability of some members to induce cell death. A new face to the TNF system seems at hand.

The TNF Receptor Family Interacts with a Parallel Family of Ligands

The receptors, with two exceptions, are all type I membrane proteins with sequence homology (almost entirely) confined to the extracellular region. The exceptions, T2 and A53R, are poxvirus gene products that map to different genetic loci and have been shown to encode soluble, secreted forms of TNF receptors (Smith et al., 1991). These function to complex (and thereby inactivate) host-produced TNF. T2 is clearly an acquired form of the p75 cellular receptor, while A53R, since it binds only TNFα and shows much lower sequence homology, may represent a third TNF receptor. The extraordinary virulence of wild-type myxoma poxvirus, uniformly fatal to its host (rabbits), is reduced nearly 50% in recombinants differing only by an inactivated *T2* gene (Upton et al., 1991). Interestingly, an intact *T2* gene is also conserved in the recently sequenced variola genome, the pathogen responsible for smallpox in humans (Shchelkunov et al., 1993).

The canonical motif of all these receptors is that of cysteine-rich pseudorepeats, each containing about six cysteines and 40 amino acids, although considerable variation in size and number is evident (e.g., CD30 and CD27). Soluble forms, released by proteolysis, for most of these receptors have been observed; one (4-1BB) is generated through alternative splicing (Goodwin et al., 1993). The cytoplasmic domains are rather small (46–221 residues) and generally lack sequence homology among themselves, suggesting major differences in signaling mechanisms. None possess sequences implying catalytic activity.

The ligands for CD30, CD27, CD40, 4-1BB, and Fas were identified and cloned not by protein sequencing, but through direct expression cloning strategies (Goodwin et al., 1993; Suda et al., 1993). This approach rested on the assumption that putative ligands would, like TNFα, exist in active surface forms identifiable by specific binding of soluble receptors (as immunoglobulin fusion chimerics). In fact, all ligands except LTα, which appears to be a secreted protein, reflect prototypic pro-TNFα architecture: type II membrane proteins, with the C-terminus extracellular, the N-terminus intracellular, and a single transmembrane element. Soluble (proteolytically released) forms of TNFα are well known, although reports have not yet established such alternative forms for other ligand members. Family-defining sequence homology (Figure 3) is restricted to ~150 residues in the C-terminal (receptor-

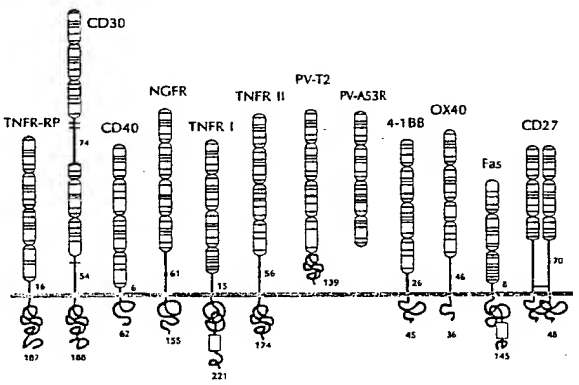

Figure 1. The TNF Receptor Superfamily

Homologous domains are shown as open boxes and cysteine residues by horizontal lines. Number of amino acids in the (nonhomologous) extracellular linker and cytoplasmic domains are indicated. Stippled boxes in the cytoplasmic regions represent death domains. TNFR-RP is a predicted family member encoded by a transcribed sequence from human chromosome 12p (Baens et al., 1993). OX40 is a rat T cell activaton antigen with no reported cognate. In laboratory strains of vaccinia virus, the A53R open reading frame is interrupted by a premature termination codon (Goebel et al., 1990). See Goodwin et al. (1993) for original references.

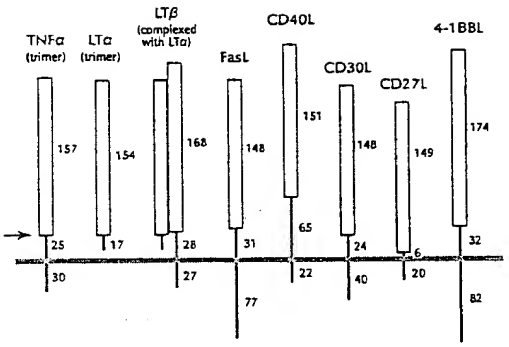

Figure 2. The TNF Family of Cytokines

The homologous C-terminal domains are indicated by open boxes. Extracellular and cytoplasmic domains, which lack sequence homology, are indicated by closed bars. The number of amino acids in each domain is shown. LTα is shown in both secreted and alternative membrane-associated forms, complexed with LTβ. The arrow indicates the proteolytic cleavage site in pro-TNFα that allows for the release of soluble form. Only TNFα, LTα, and LTβ have been shown to form oligomers.

Figure 4. Crystal Structure of Soluble p55 TNFR–LTα Complex

Reprinted from Figure 4 in Banner et al. (1993).

binding) region, which in soluble TNFα and LTα fold into a β-pleated sheet sandwich and trimerize (Eck et al., 1992; Jones et. al., 1989). Sequence conservation is particularly high at protomer interfaces. It seems likely, therefore, that all ligands in this family adopt a similar tertiary structure and form oligomers.

A unifying picture of the prototypic interation between ligands and receptors has literally crystallized from a milestone X-ray diffraction study by Banner et al. (1993), who solved the structure of a human LTα-soluble p55 TNF receptor complex. This complex, containing the extracellular portions of three receptors bound to one LTα trimer, clearly establishes the pseudorepeat sequences in the receptor as true domains forming an elongated array that lies in the interfaces between each pair of the three ligand protomers (Figure 4). Roughly 80% of receptor–ligand contacts occur through domain 2, and each receptor contacts both protomers in the interface. Such a complex would bring receptor cytoplasmic domains into close apposition, presumably complementing binding sites for unknown signaling components, and is consistent with ligand-induced receptor cross-linking as the near universal activation mechanism for growth factors. The novel feature here is that activation involves receptor trimerization; most cytokine families appear to induce dimerization, although by different schemes. Platelet-derived growth factor, for example, is a dimer (immunoglobulin family) whose receptor is a tyrosine kinase, while growth hormone, a member of the hematopoietin family, is a heterodivalent monomer (De Vos et al., 1992).

Many observations, however, suggest this disarmingly simple picture may need revisions. First, structural divergencies in other family members imply variations in interaction motifs. CD30, for example, contains six domains, not four, separated by a nonhomologous region of 74 residues, while CD27 contains three domains, one truncated, and appears to be a disulfide-linked dimer. Further, some evidence suggests that TNFα and LTα oligomers may be intrinsically polydisperse, consisting of homodimers, trimers, and tetramers (Schoenfeld et al., 1990).

Second, one ligand family member (LTβ), with no known biological activity, has been shown to form heterologous complexes with mature LTα (e.g, β2α1), serving to anchor

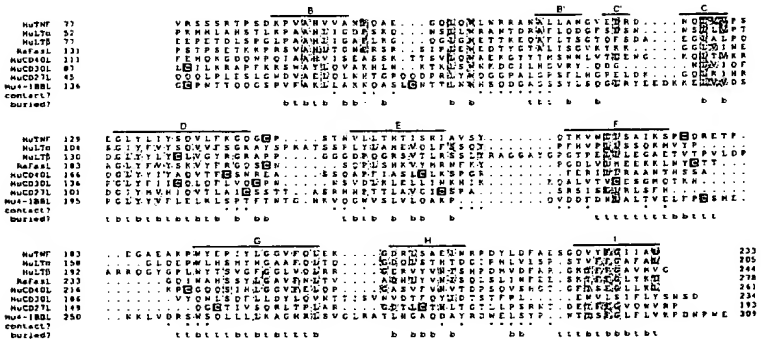

Figure 3. Sequence Homology in C-Terminal Domains of TNF Ligand Family

Alignment begins with N-terminus of soluble TNFα. Residues conserved in four or more members are stippled; cysteines are in closed boxes. Letters B–I indicate β strands in TNF tertiary structure. Asterisks indicate residues having p55 receptor contacts in crystal structure. Lowercase b indicates buried residues in the β-sheet interior; t, residues at TNF protomer–protomer interfaces.

the normally secreted LTα to the cell surface of T, B, and NK cells (Browning et al., 1993). Superficially, these ligands thus begin to resemble cassettes, whereby combinatorial arrangements could produce different oligomers with potentially altered receptor specificities, greatly increasing diversity of function. Such a mechanism in an entirely different family is reflected by the use of the platelet-derived growth factor A and B ligand subunits (A–A, A–B, and B–B dimers) to generate heterotypic and homotypic cross-linked dimers of the α and β receptors. Generic ligand–receptor interactions suggested by the crystal structure of LTα–p55, however, predict that mixed oligomers of LTα–LTβ would produce inactive (that is, ligand-bound but un-cross-linked) p55 (or p75) TNF receptor complexes. This suggests one function of LTβ is to inactivate LTα (with respect to TNF receptors) and implies the existence of a distinct receptor for LTβ that, when cross-linked, would generate novel signals. Heterologous complexes with still other family members could enormously increase the complexity of biological networks.

Third, there is no clear rationale for the redundancy built into the TNFα/LTα (p55/p75) system, nor is it clear how common such redundancies will be in other family members. These ligands display nearly identical biological activities and bind each receptor. Most cells, however, express variable levels of both receptors, even though heterologous receptor cross-links seem prohibited and each receptor can, on its own, transduce different signals (Pfeffer et al., 1993; Tartaglia et al., 1993). One implication is that functional cross-talk may exist between family members.

Fourth, the cytoplasmic domains of these ligands clearly serve important but unknown functions: they are nearly as conserved in sequence across species as extracellular regions, suggesting they carry binding sites for unidentified proteins. For example, we calculate the cytoplasmic domains of human and mouse CD40L are 82% identical; mouse and human TNFα, 86%; mouse and human CD30L, 61%. There is little homology among these domains in different ligands, however, arguing against conservation as a result of common biosynthetic or internalization mechanisms. Since direct cell–cell contact is a primary means of ligand–receptor interaction in this family, bipolar signaling may occur, blurring the distinction between receptor and ligand.

Finally, the low affinity nerve growth factor receptor (NGFR), while structurally a member of this family, binds a family of ligands (the neurotropins) structurally rather different than TNF. NGFR also interacts with the *trk* family of receptor tyrosine kinases, which show no homology to TNF receptors. Further, while the genomic architectures of CD40, both TNF receptors, and CD27 are rather similar, they appear quite different from the p75 NGFR. Thus, whether the (extracellular) structural homology of NGFR subunit reflects a functional interaction or even distant evolutionary relatedness between these two systems is unclear (Smith et al., 1993). Intriguingly, however, NGF has been shown to affect lymphocyte function, NGFRs are expressed at high levels on follicular dedritic cells in germinal centers, and TNF receptors are expressed on glial cells of the nervous system, raising the possibility of functional interplay.

Biological Netw rks: Ap ptosis, Necr sis, and Costimulation

Ligand family members can induce pleiotropic biological responses, including differentiation, proliferation, activation, or even cell death. It is clear, however, that T cell–mediated immunity, particularly contact dependent and antigen driven, provides one unifying theme. Without exception, all ligands as well as their receptors are T cell products (although not uniquely so). Both human PBT cells and $CD4^+$ T cell clones show enhanced proliferation when treated with any family ligand in the presence of monoclonal antibodies to CD3 (Goodwin et al., 1993). Thus, autocrine T cell loops, largely mediated through cell–cell contact, are a common feature of the family. The observed variation in ligand induction kinetics is also consistent with different roles for these ligands in T cell activation (Smith et al., 1993; Beutler, 1992). As B cells are also capable of expressing receptors for CD30, CD40, TNFα, LTα, and CD27, for example, this family may contribute T cell help to B cells as well. TNFα and CD30L, however, are also abundantly expressed by activated macrophages, with receptors for the former expressed on nearly all vertebrate cells. Clearly, the dominant physiological networks are an evolving subject.

A consequence of the type II membrane protein architecture of these ligands, particularly on T and B cells, is the cell–cell contact nature of the interaction: it helps ensure ligand expression is antigen dependent and demands that signals generated by TNF family ligands in target cells are productively coordinated with accessory signals generated by other cognate pairs (e.g., cytokine–receptors, CD80–CD28, adhesion molecules such as CD58–CD2). The essence of signaling in this family is therefore one of costimulation. Soluble forms of TNFα (or perhaps CD30L) produced by macrophage may serve to extend the range of activities and provide flexibility to the immune response (Browning et al., 1993; Smith et al., 1993).

The biological function of CD40–CD40L provides a particularly clear example of costimulatory function. Almost exclusively the product of activated $CD4^+$ T cells, CD40L provides essential signals to purified B cells, costimulated with interleukin-4, to undergo immunoglobulin isotype switching and to secrete mature immunoglobulin. Confirmation of this in vitro picture comes from studies of patients carrying mutations in the X-linked CD40L gene: patients show normal numbers of B cells, but a virtual absence of immunoglobulin isotypes other than immunoglobulin M and an inability to mount an antigen-specific antibody response, with concomitant susceptibilty to opportunistic infections (Callard et al., 1993).

The TNFα/LTα (p55/p75) system is more complex. Transgenic mice deleted of the p55 TNF receptor illuminate the fundamental importance of TNF and this receptor while illustrating the difficulty in unraveling networks in such a pleiotropic system (Pfeffer et al., 1993). These animals are severely impaired in the clearance of the bacterial pathogen Listeria monocytogenes, die rapidly from infections, and are extraordinarily resistant to lipopolysaccha-

ride-mediated septic shock. Lymphocyte populations, however, are normal and clonal deletion of potentially self-reactive T cells is unimpaired, indicating normal thymocyte development. The creation of p75 TNFR knockout transgenics and, particularly, of double knockouts of p75 and p55 should prove invaluable in dissecting this complex system.

The most recently cloned family member is the Fas ligand, the search for which had approached the status of an immunological Holy Grail (Suda et al., 1993). The reason is that the Fas antigen, broadly expressed on both myeloid and lymphoid cells, including thymocytes, has been characterized as responding to activation (through cross-linking) by inducing apopotic (programmed) cell death. Since this process is fundamental to immune system development and Ca^{2+}-independent T cell–mediated cytotoxicity, the ligand may play a crucial role in these phenomena. This is consistent with the finding that a naturally occurring autosomal recessive mutation in mice, *lpr* (lymphoproliferation), maps to the *fas* locus, and homozygous animals exhibit lymphadenapathy and autoimmune disease, resembling systemic lupis in humans. One *lpr* mutant producing a defective Fas protein behaves as a dominant-negative mutation with respect to a phenotypically very similar mutation, *gld* (generalized lymphoproliferation disease), on a different chromosome, suggesting that *fas* and *gld* encode receptor–ligand cognates (Allen et al., 1990). Nagata and colleagues demonstrate that this is indeed the case (Takahashi et al., 1994 [this issue of *Cell*]). While defects in the Fas system clearly give rise to aberrancies in the immune system, it seems the proximal cause is not thymic failure to eliminate autoreactive clones through apoptosis, as originally suggested (Watanabe-Fukunaga et al., 1992). Indeed, T cell repertoire formation, as well as both positive and negative selection, proceeds normally in homozygous *lpr* (or *gld*) animals (Sidman et al., 1992). Instead, Fas appears to be involved in activation-driven T cell suicide, a process by which chronically activated mature T cells undergo apoptosis, suggesting a role for Fas in peripheral tolerance (Russel and Wang, 1993).

The contingent ability to induce death is rather unique to this family and is well established for TNFα, LTα, and FasL. The p55 TNFR and Fas share a 65 residue homology region in the cytoplasmic domains, which deletion studies have established to be crucial for the apoptotic death activity (Takahashi et al., 1994; Tartaglia et al., 1993). However, Fas appears to have a pleiotropic nature, and it is here that Fas mirrors the properties of other family members. Fresh PBT cells, for example, as well as some B cell tumors, respond to Fas activation with proliferation, not death (Mapara et al., 1993; Alderson et al., 1993). Strikingly similar is the behavior of at least three other family members, CD30 and both TNF receptors. In each case, the specific responses, including cell death (apoptotic or necrotic) or proliferation, depend upon cell type, stage of differention, transformation status, and the presence of other stimuli. Thus, elucidation of the full spectrum of FasL activities may prove unexpectedly illuminating. Their fundamental involvement in the immune system and the window they provide on the apoptosis phenomena, crucial to many aspects of vertebrate development and homeostasis, combine to ensure ever greater inter st in this emerging cytokine family.

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